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(71) Applicant: Takeda Chemical Industries, Ltd.  
27, Doshomachi 2-chome Higashi-ku  
Osaka-shi Osaka, 541(JP)

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(72) Inventor: Nishimura, Osamu  
123-502, 2 Higashitoyonakacho 5-chome  
Toyonaka Osaka 560(JP)

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(72) Inventor: Fujino, Masahiko  
10-7, Hieberigaoka 2-chome  
Takarazuka Hyogo 665(JP)

(74) Representative: von Kreisler, Alek, Dipl.-Chem. et al.  
Deichmannhaus am Hauptbahnhof  
D-6000 Köln 1(DE)

(54) Chemically modified lymphokine and production thereof.

(56) The invention provides chemically modified lymphokines having  $R-O-CH_2-CH_2-$ , wherein R is a protective group for the terminal oxygen and n is an optional positive integer, bonded directly to at least one primary amino group of the lymphokine moiety, and a method of producing the same.

The chemically modified lymphokines according to the invention can be produced by reacting a lymphokine with an aldehyde of the formula  $R-O-CH_2-CH_2-$ , O-CH<sub>2</sub>CHO where R and n are as defined above, in the presence of a reducing agent.

The chemically modified lymphokines according to the invention are useful as drugs, among others.

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Chemically Modified Lymphokine and Production Thereof

Lymphokines such as interferons (hereinafter sometimes abbreviated as IFNs) and interleukin-2 (hereinafter sometimes abbreviated as IL-2) have been considered to be of clinical value for the treatment of viral infections and malignancies 5 and recent technological advances in genetic engineering have made it in principle possible to produce such lymphokines on large scales. However, it is known that the clearance of lymphokines administered to the living body is in general very short. In the case of lymphokines derived from heterologous 10 animals, it is anticipated that antibodies may be produced in some instances and cause severe reaction such as anaphylaxis. Therefore, technology development is desired which leads to delayed clearance of lymphokines used as drugs, with their activity retained, and further to decrease in their 15 antigenicity. To achieve this object, chemical modification of lymphokines is a very effective means. Such chemical modification is expected to result in delayed clearance in the living body, decreased antigenicity and, further, increased physiological activiy. From the practical viewpoint,

the significance of chemical modification of lymphokines  
is thus very great.

Generally, in chemically modified physiologically active proteins, a method is required by which said proteins 5 can be chemically modified while retaining their physiological activity. Polyethylene glycol methyl ether is considered to have no antigenicity and therefore is used in chemical modification of proteins. The introduction of said substance into proteins is generally performed by way of the intermediary 10 of cyanuric chloride. However, cyanuric chloride is toxic per se and the possible toxicity of its degradation products in vivo remains open to question. Therefore, cyanuric chloride should be used with caution. Furthermore, the reaction involved requires a pH on the alkaline side and therefore 15 the above-mentioned method of modification has a drawback in that it cannot be applied to proteins liable to inactivation under alkaline conditions.

U. S. Patent No. 4,002,531 discloses a method of producing monoalkylpolyethylene glycol derivatives of enzymes. However, 20 the method disclosed therein, which uses sodium borohydride at pH 8.5, when applied to lymphokines, may possibly destroy the physiological activity of lymphokines and therefore cannot serve as an effective method of production. Furthermore, said patent specification does not any suggestion as to 25 the effect of delaying the in vivo clearance of the enzyme derivatives. Such effect is therefore unknown.

There is also known a method of introducing a low

molecular aldehyde such as formaldehyde, acetaldehyde, benzaldehyde or pyridoxal into physiologically active proteins in the presence of a boron-containing reducing agent [Methods in Enzymology, 47, 469-478 (1977); Japanese Patent Unexamined 5 Publication No. 154,596/83]. However, application of said method to lymphokines fails to achieve effective delay in clearance. A substantial decrease in antigenicity cannot be expected but rather it is possible that the low molecular aldehyde introduced may serve as a hapten to thereby provide 10 said lymphokines with immunogenicity.

The present inventors studied intensively to overcome the above difficulties and have now completed the present invention.

This invention provides chemically modified lymphokines 15 having polyethylene glycol of the formula  $R-(O-CH_2-CH_2)_n$  (I) wherein R is a protective group for the terminal oxygen atom and n is an optional positive integer, bonded directly to at least one primary amino group of the lymphokine moiety and a method of producing the same.

20 In the present specification, the term "lymphokine" includes soluble factors released from lymphocytes and involved in cellular immunity and substances equivalent thereto in physiological activity.

Thus, the lymphokines may be genetically engineered 25 products, products derived from various animals including humans and further include substances similar in structure and in physiological activity to these.

For instance, there may be mentioned various interferons [interferon- $\alpha$  (IFN- $\alpha$ ), interferon- $\beta$  (IFN- $\beta$ ), interferon- $\gamma$  (IFN- $\gamma$ ),], IL-2, macrophage differentiating factor (MDF), macrophage activating factor (MAF), tissue plasminogen activator, 5 and substances similar in structure and in physiological activity to these.

Examples of said substances similar in structure and in physiological activity are substances having the structure of IFN- $\gamma$  except for the lack of 2 to 4 amino acids at the 10 N-terminal thereof (PCT/JP84/00292, filed June 6, 1984), various IFN- $\gamma$  fragments lacking in the C terminal portion of IFN- $\gamma$  (e.g. 15K species; EPC Patent Application No. 84 111133.9 ), substances having the structure of IL-2 except for the lack of the N-terminal amino acid 15 thereof (EPC (laid open) 91539) or the lack of 4 amino acids from the N-terminal (Japanese Patent Application 58-235638, filed December 13, 1983) and substances having the structure of IL-2 except for the lack of one or more constituent amino acids with or without one or more substitute amino acids 20 in place of said missing one or ones, for example the IL-2 analog containing serine in lieu of the 125th amino acid cysteine (EPC (laid open) 104798).

Preferred among such lymphokines are IFN- $\alpha$ . IFN- $\gamma$  (consisting of 146 amino acids (EPC (laid open) 0089676)), 25 IFN- $\gamma$  lacking in two N-terminal amino acids (IFN- $\gamma$  d2), IFN- $\gamma$  lacking in three N-terminal amino acids (IFN- $\gamma$  d3),

and IL-2.

The lymphokines to be used in the practice of the invention preferably have a molecular weight of 5,000 to 50,000, more preferably 10,000 to 30,000.

5       The primary amino group of lymphokines includes the N-terminal  $\alpha$ -amino group and the  $\epsilon$ -amino group of the lysine residue.

Referring to the group represented by the above formula (I), the terminal oxygen-protecting group R is, for example, 10 an alkyl or alkanoyl group. The alkyl group is preferably an alkyl of 1 to 18 carbon atoms, more preferably a lower ( $C_{1-4}$ ) alkyl, such as methyl, ethyl, propyl, i-propyl, butyl, i-butyl, sec-butyl or t-butyl. The alkanoyl group is preferably an alkanoyl of 1 to 8 carbon atoms, more preferably a 15 lower ( $C_{1-6}$ ) alkanoyl, such as formyl, acetyl, propionyl, butyryl, i-butyryl or caproyl. The positive integer n is preferably not more than 500, more preferably 7 to 120.

The group of formula (I) preferably has a molecular weight of not more than 25,000, more preferably 350 to 6,000. 20 From the viewpoints of physiological activity retention and clearance delaying effect, the group of formula (I) preferably has a molecular weight corresponding to 1 to 10%, more preferably 2 to 5% of the molecular weight of the lymphokine to be modified.

25       The chemically modified lymphokines according to the invention have the group of formula (I) directly bonded to at least one of the primary group of the corresponding

lymphokines.

When the N-terminal  $\alpha$ -amino group is the only primary amino group in the lymphokine to be modified, the modified lymphokine has the group of formula (I) directly bonded to said amino group. When the lymphokine to be modified has one or more lysine residues in its molecule, the modified lymphokine has the group of formula (I) directly bonded to some percentage, preferably 15 to 80% (on the average), of said  $\epsilon$ -amino groups. In this case, the N-terminal  $\alpha$ -amino group may have or may not have the group of formula (I) directly bonded thereto.

The chemically modified lymphokines according to the invention can be produced, for example, by reacting a lymphokine with the aldehyde of the formula  
15  $R \leftarrow O-CH_2-CH_2 \rightarrow_{n-1} O-CH_2-CHO$  (II) wherein R and n are as defined above, in the presence of a reducing agent.

As the boron-containing reducing agent to be used as conducting the reaction, there may be mentioned sodium boro-hydride and sodium cyanoborohydride. Among them, more preferred is sodium cyanoborohydride from the viewpoint of selectivity of reaction or possibility of carrying out the reaction in the neighborhood of neutrality.

In carrying out the reaction, the aldehyde (II) is used in an amount of about 1 to 10,000 moles per mole of the lymphokine, and the boron-containing reducing agent is used in an amount of about 1 to 100 moles per mole of the lymphokine. The degree of modification can be selected as desired by varying the mole ratio between lymphokine and aldehyde

(II). The solvent to be used in carrying out the invention may be any solvent which does not disturb the reaction and is, for example, a buffer such as a phosphate or borate buffer. An organic solvent which does not inactivate lymphokines or disturb the reaction, such as a lower alkanol (e.g. methanol, ethanol, i-propanol) or acetonitrile, may be added.

5 The reaction may be conducted within a broad pH range of 3 to 14 but is preferably performed in the vicinity of neutrality (pH 6.5-7.5). The reaction temperature may be selected within a broad range of 0° to 80°C, preferably 0° to 50°C,

10 so as not to cause denaturation of lymphokines. A period of 0.5 to 100 hours, generally 10 to 80 hours, will be sufficient for the reaction. The desired, chemically modified lymphokines can be obtained by purifying the reaction mixture

15 by dialysis, salting out, ion exchange chromatography, gel filtration, high performance liquid chromatography, electrophoresis, or the like ordinary method of purifying proteins. The degree of modification of the amino group or groups can be calculated by acid degradation followed by amino acid analysis, for

20 instance.

The above-mentioned aldehyde (II) can be produced from an ethylene glycol derivative of the formula R- $\text{O}-\text{CH}_2\text{CH}_2-\text{n}-\text{OH}$  (III) wherein R and n are as defined above, for instance. The following is a method of

25 producing the same which is advantageous in that the production of the corresponding byproduct carboxylic acid is little.

Thus, the compound (III) is oxidized with pyridinium chlorochromate in a haloalkane solvent such as methylene chloride or chloroform. In this case, pyridinium chlorochromate is used in an amount of 1 to 3 moles per mole of compound (III) and the reaction is carried out at -10° to 50°C, preferably at room temperature, for 1 to 30 hours.

Treatment of compound (III) (n-1) with potassium t-butoxide in t-butanol followed by reaction with a bromoacetal and treatment with an acid such as an organic acid (e.g. trifluoroacetic acid) or an inorganic acid (e.g. hydrochloric or sulfuric acid) can also give the corresponding aldehyde (II) which is longer in chain length by  $-O-CH_2CH_2-$  than compound (III). In this case, 10 to 30 moles, per mole of compound (III), of potassium t-butoxide is added to the above compound and, after dissolution, 3 to 15 moles, per mole of compound (III), of a bromoacetal is added, followed by reaction at 10° to 80°C for 0.5 to 5 hours. After treatment of the reaction mixture in the conventional manner, the product is dissolved in a dilute aqueous solution of the above-mentioned acid, followed by heating for 5 minutes to 2 hours.

In each case, the reaction mixture can be subjected to purification process conventional in the field of chemistry, such as extraction, concentration, recrystallization, re-precipitation, chromatography and/or distillation.

The chemically modified lymphokines according to the invention have useful physiological activities similar to

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those of the corresponding known, unmodified lymphokines and are useful as drugs, among others.

The chemically modified lymphokines according to the invention exhibit delay in clearance in vivo as compared with 5 the corresponding known, unmodified lymphokines and are low in toxicity and antigenicity and can be used safely for the same purposes and in the same manner as in the case of known lymphokines.

The chemically modified lymphokines according to the 10 invention can usually be administered to mammals (monkey, dog, pig, rabbit, mouse, human) either orally or parenterally in the form of appropriate pharmaceutical compositions prepared by using carriers, diluents, etc., which are known in themselves.

15 Thus, for instance, chemically modified IFN- $\alpha$  according to the invention, when used as an antiviral agent, is recommendably administered to human adults once a day by intravenous injection in a dose of  $1 \times 10^4$  to  $1 \times 10^9$  international units.

20 In the present specification, the amino acids, when referred to by abbreviations, are abbreviated according to IUPAC-IUB (Commission of Biological Nomenclature).

The transformant Escherichia coli 294/pHITtrpl101-d2 as disclosed hereinlater in a reference example has been 25 deposited with Institute for Fermentation, Osaka (IFO) under the deposit number IFO-14350 and, since June 6, 1984, with the Fermentation Research Institute (FRI), Agency of Industrial Science and Technology.

Ministry of International Trade and Industry under the deposit number FERM BP-703 under Budapest Treaty.

The strain Escherichia coli DH1/pTF4 has been deposited with the Institute for Fermentation, Osaka under the deposit number IFO-14299 and, since April 6, 1984, with the FRI under 5 the deposit number FERM BP-628 under Budapest Treaty.

#### BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows the clearance-delaying effect in rat plasma as disclosed in Example 1 (iv). The measurement results obtained with the chemically modified IFN- $\alpha$  according to the invention as produced in Example 1 (i) are indicated by O (enzyme immunoassay) and  $\square$  (antiviral activity assay), and the results obtained with rIFN- $\alpha$ A used as a control by  $\bullet$  (enzyme immunoassay) and  $\blacksquare$  (antiviral activity assay).

15 Fig. 2 shows the clearance-delaying effect in rat plasma as disclosed in Example 3 (ii). The data indicated by  $\Delta$ ,  $\square$  and  $\bullet$  are the enzyme immunoassay data for compound No. 8, compound No. 2 (Table 1) and control rIFN- $\alpha$ A, respectively.

Fig. 3 shows the construction scheme for the expression 20 plasmid pHITtrp1101-d2 disclosed in Reference Example 3 (i) and Fig. 4 the construction scheme for the expression plasmid pLC2 disclosed in Reference Example 4 (i).

#### BEST MODE FOR CARRYING OUT THE INVENTION

The following working examples and reference examples 25 illustrate the invention in more detail but are by no means limitative of the invention.

Example 1 Production of polyethylene glycol methyl ether-modified IFN- $\alpha$

(i) A 5-ml (4.8 mg as protein) portion of a solution of IFN- $\alpha$  (rIFN- $\alpha$ A) was dialyzed against 0.2 M phosphate buffer (pH 7.0) and 0.15 M sodium chloride at 4°C for 12 hours. To the dialyzate taken out, there was added the polyethylene-glycol methyl ether aldehyde (average molecular weight 1,900) (260 mg) obtained in Reference Example 1. Then,

sodium cyanoborohydride (140 mg) was added, and the mixture was stirred at 37°C for 40 hours. The reaction mixture was poured into a Sephadex G-75 column (3.0 x 43.0 cm) and developed with 25 mM ammonium acetate buffer (pH 5.0) and 5 0.15 M sodium chloride. The eluate was collected in 5-ml portions. Eluate fractions (100-150 ml) containing the contemplated product were combined. Assaying by the Lowry method using bovine serum albumin as a standard revealed that the protein content in the combined fractions 10 was 84 ug/ml. Amino acid ratios in acid hydrolysate (6 N hydrochloric acid, 110°C, 24 hours) were as follows: Asp, 12.2 (12); Thr, 10.4 (10); Ser, 16.0 (14); Glu, 24.8 (26); Pro, 6.0 (5); Gly, 6.3 (5); Ala, 8.6 (8); Val, 6.5 (7); Met, 4.0 (5); Ile, 7.6 (8); Leu, 21.0 (21); Tyr, 5.2 (5); Phe, 9.9 (10); Lys, 6.5; His, 3.8 (3); 15 Arg, 9.1 (9); Cys, Trp, decomposed. In view of the fact that rIFN- $\alpha$ A contains 11 Lys residues, the above results led to a conclusion that about 41% of Lys residues in interferon  $\alpha$  had been modified at the  $\epsilon$ -amino group with the 20 polyethylene glycol methyl ether. (average molecular weight 1,900). The potency of this product as determined by the enzyme immunoassay method [Methods in Enzymology, 79, 589- 595 (1981)] was  $1.51 \times 10^7$  international units/mg and the antiviral activity as determined by the method described 25 in Journal of Virology, 37, 755-758 (1981) was  $0.57 \times 10^7$  international units/mg. This product (IFA-3) was submitted

to a clearance test in rats as mentioned later herein.

(ii) Using 100 mg of the polyethylene glycol methyl ether aldehyde obtained in Reference Example 1 and having an average molecular weight of 750 and 100 mg of sodium cyanoborohydride, rIFN- $\alpha$ A was treated in the same manner as (i) to give 30 ml of a solution of polyethylene glycol methyl ether-modified IFN- $\alpha$  with a protein content of 130  $\mu$ g/ml.

5 Amino acid ratios in acid hydrolysate

(6 N hydrochloric acid, 110°C, 24 hours) were as

10 follows: Asp, 12.1 (12); Thr, 10.1 (10); Ser, 13.6 (14); Glu, 26.7 (26); Pro, 5.5 (5); Gly, 5.6 (5); Ala, 8.4 (8); Val, 6.7 (7); Met, 5.5 (5); Ile, 7.4 (8); Leu, 21.0 (21); Tyr, 5.1 (5); Phe, 9.6 (10); Lys, 4.7; His, 3.5 (3); Arg, 9.1 (9); Trp, 1.8 (2); Cys, decomposed. The above data  
15 indicate that about 57% of Lys residues had been modified at the  $\epsilon$ -amino group. Enzyme immunoassay performed in the same manner as (i) gave the result  $5 \times 10^6$  international units/mg, and the antiviral activity of the product was  $0.14 \times 10^8$  international units/mg.

20 (iii) The procedure of (i) was followed using 27 mg of the polyethylene glycol methyl ether aldehyde and 27 mg of sodium cyanoborohydride and there was obtained 50 ml of a polyethylene glycol methyl ether-modified IFN- $\alpha$  solution with a protein content of 45  $\mu$ g/ml. Amino acid ratios in  
25 acid hydrolysate (6 N hydrochloric acid, 110°C, 24 hours) gave the following results: Asp, 13.6 (12); Thr,

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10.4 (10); Ser, 14.9 (14); Glu, 26.6 (26); Pro, 5.5 (5);  
Gly, 6.1 (5); Ala, 8.3 (8); Val, 6.6 (7); Met, 5.2 (5);  
Ile, 7.4 (8); Leu, 21.0 (21); Tyr, 5.3 (5); Phe, 10.2 (10);  
Lys, 9.0; His, 3.6 (3); Arg, 9.1 (9); Trp, 2.3 (2); Cys,  
5 decomposed. The above data indicate that about 18% of Lys  
residues had been modified at the ε-amino group. Enzyme  
immunoassay performed in the same manner as (i) gave the  
result  $1.09 \times 10^8$  international units/mg and the antiviral  
activity of this product was  $1.53 \times 10^8$  international units/mg.  
10 (iv) The chemically modified IFN-α (IFA-3) of the invention  
as obtained above in (i) was administered to a group  
of three 7-week-old female SD rats by injection into the  
femoral muscle in a dose of  $1.274 \times 10^6$  units per capita.  
After a prescribed period, blood was sampled from the caudal  
15 vein and the IFN-α potency in plasma was determined by the  
enzyme immunoassay method and antiviral activity method  
described in Example 1 (i). A distinct delay in clearance  
was observed as compared with a group administered unmodi-  
fied interferon α (rIFN-αA) in a dose of  $1.259 \times 10^6$  units  
20 per capita.

The above results are depicted in Fig. 1.

Example 2

To 5 ml of the solution of chemically modified IFN-α  
(IFA-3) of the invention as obtained in Example 1 (i),  
25 there is added 250 mg of human serum albumin. The resulting  
solution is filtered through a membrane filter (pore size:

0.2  $\mu\text{m}$ ) and distributed into 5 vials, followed by lyophilization and storage. The contents of each vial are dissolved in 1 ml of distilled water for injection just prior to use.

Example 3 Production of polyethylene glycol methyl ether-

5 modified IFN- $\alpha$  and alkanoyl-polyethylene glycol-modified IFN- $\alpha$

(i) The title compounds were synthesized by using the polyethylene glycol methyl ether aldehyde and alkanoyl-polyethylene glycol aldehyde obtained in Reference Example 10 1 and Reference Example 2, respectively, and following the procedure of Example 1. Various data for each derivative synthesized are shown in Table 1 and amino acid analysis data therefor in Table 2.

(ii) The chemically modified IFN- $\alpha$  species obtained in (i) 15 above (compounds No. 2 and No. 8) were administered to 7-week-old female SD rats in groups of 3 by intramuscular injection into the femur in doses of  $3.12 \times 10^6$  units and  $2.66 \times 10^6$  units, respectively. Thereafter, blood samples 20 were collected from the caudal vein at times intervals and assayed for IFN- $\alpha$  potency in plasma by enzyme immunoassay. Obviously delayed clearance was noted as compared with the group given  $3.82 \times 10^6$  units of unmodified IFN- $\alpha$ . These results are depicted in Fig. 2.

Table 1 Polyethylene glycol methyl ether-modified interferon  $\alpha$  and alkanoyl polyethylene glycol-modified interferon  $\alpha$ 

Compound No.	IFN- $\alpha$ amount	PEG aldehyde (Av. mol. wt.)	Reaction temp.	PEG aldehyde amount (mg)	Addition of NaBH <sub>3</sub> CN	NaBH <sub>3</sub> CN amount (mg)	Reaction time (hours)	Content OD 200nm (mL)	Obtained yield (%)	EIA	AVA
1	5 $\mu$ l (4 . 2mg)	MeOP EG (5000)	37 (ca. 20 times)	252 (ca. 20 times)	Sane time	50 (ca. 200 times)	18	0.139	30	99	31
2	5 $\mu$ l (4 . 2mg)	MeOP EG (5000)	37 (ca. 10 times)	124 (ca. 10 times)	Sane time	54 (ca. 200 times)	10	0.151	22	79	18
3	5 $\mu$ l (4 . 2mg)	MeOP EG (5000)	37 (ca. 5 times)	61 (ca. 5 times)	Sane time	52 (ca. 200 times)	18	0.210	20	100	3.6
4	5 $\mu$ l (4 . 2mg)	MeOP EG (1900)	37 (ca. 10 times)	47 (ca. 10 times)	3 hrs later	50 (ca. 200 times)	18	0.175	17.5	73	1.3
5	5 $\mu$ l (4 . 2mg)	MeOP EG (750)	4 (ca. 60 times)	110 (ca. 70 times)	5 hrs later	50 (ca. 200 times)	24	0.100	36	84	51
6	5 $\mu$ l (4 . 2mg)	MeOP EG (550)	4 (ca. 70 times)	96 (ca. 70 times)	24 hrs later	100 (ca. 400 times)	48	0.117	25	70	46
7	5 $\mu$ l (4 . 2mg)	MeOP EG (350)	4 (ca. 120 times)	102 (ca. 120 times)	5 hrs later	100 (ca. 400 times)	70	0.107	36	91	79
8	5 $\mu$ l (4 . 2mg)	Acetyl PEG (1540)	4 (ca. 50 times)	104 (ca. 50 times)	7.5 hrs later	60 (ca. 240 times)	24	0.150	25	89	40
9	5 $\mu$ l (4 . 2mg)	Caproyl PEG (1100)	4 (ca. 50 times)	120 (ca. 50 times)	9 hrs later	50 (ca. 200 times)	24	0.007	35	73	56

PEG: Polyethylene glycol, MeOP EG: Polycethylene glycol methyl ether.

The value in parentheses is the average molecular weight.

NaBH<sub>3</sub>CN: Sodium cyanoborohydride, EIA: Enzyme immunoassay, AVA: Antiviral activity

Table 2

Com- ound No.	Amino acid analysis value										Theo- retical -α A
	1	2	3	4	5	6	7	8	9	rIFN	
Asp	12.8	12.7	12.5	12.5	13.4	12.9	12.2	12.5	12.8	12.6	12
Thr	11.7	11.6	11.2	10.9	11.3	11.4	10.9	11.6	11.3	11.6	10
Ser	15.8	16.7	15.7	15.4	17.6	15.6	15.4	16.8	15.6	15.6	14
Glu	27.4	27.0	26.7	27.3	27.8	27.3	26.1	26.3	26.4	27.6	26
Pro	-	5.3	5.6	5.5	5.6	5.8	5.5	5.7	5.7	3.7	5
Gly	4.9	5.0	4.6	4.6	7.1	4.6	4.5	5.3	5.4	4.6	5
Ala	8.1	8.0	8.1	7.8	8.6	7.5	7.3	8.3	8.4	7.8	8
Cys	-	-	-	-	-	-	-	-	-	-	4
Val	6.8	6.8	6.7	6.6	7.3	6.7	6.3	6.9	7.1	6.6	7
Met	3.2	4.7	4.3	4.3	4.4	4.3	4.1	4.7	4.8	3.9	5
Ile	7.7	7.7	7.7	7.6	8.0	7.6	7.3	7.5	7.6	7.6	8
Leu	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21
Tyr	4.3	4.5	4.6	4.6	4.9	4.6	4.4	4.8	4.8	4.6	5
Phe	9.8	9.8	9.8	9.8	9.8	9.8	9.4	9.7	9.8	9.8	10
Lys	8.6	10.3	10.6	9.6	5.4	6.1	2.3	6.6	4.9	11.3	11
His	2.7	3.0	2.7	2.7	2.9	2.8	2.6	2.9	2.9	4.1	3
Arg	8.8	8.8	9.2	8.8	9.1	8.8	8.5	7.7	7.6	8.9	9
Trp	-	-	-	-	-	-	-	0.8	1.0	-	2

- : Not detected.

Example 4 Production of polyethylene glycol methyl ether-modified interferon- $\gamma$

(i) A 5-ml portion (5.95 mg as protein) of a solution of the interferon- $\gamma$  protein produced by the recombinant DNA technique (hereinafter abbreviated as rIFN- $\gamma$ ; cf. EPC laid open No. 110044) was applied to a Sephadex G-25 column (2.0 x 60.0 cm) and developed with 0.2 M phosphate buffer (pH 7.0). The eluate was fractionated in 5-ml portions. Fractions Nos. 11-13 were combined and diluted to 100 ml with the same buffer. Thereto was added polyethylene glycol methyl ether aldehyde (average molecular weight 750) (225 mg), followed by addition of sodium cyanoborohydride (300 mg). The mixture was shaken at 37°C for 72 hours. The resulting precipitate was removed by centrifugation. The supernatant was concentrated to 10 ml using a Diaflow membrane (Amicon). The concentrate was applied to a Sephadex G-75 column (3.0 x 43.0 cm) and developed with 25 mM ammonium acetate buffer (pH 6.0) + 0.15 M sodium chloride + 10 mM glutathione. The eluate was fractionated in 5-ml portions. Fractions Nos. 17-24 containing the desired product were combined. The protein content in the combined fractions as determined by the Bradford method using bovine serum albumin as a standard was 7.73  $\mu$ g/ml. The acid hydrolysate (6 N hydrochloric acid, 110°C, 24 hours) gave the following amino acid analysis values: Asp, 19.6 (20); Thr, 4.7 (5); Ser, 8.3 (11); Glu, 18.5 (18); Pro, 2.1 (2); Gly, 5.4 (5); Ala, 7.5 (8); Val, 8.4 (8); Met, 3.7 (4); Ile, 7.1 (7); Leu, 9.7 (10),

Tyr, 5.3 (5); Phe, 9.7 (10); Lys, 17.6; His, 2.0 (2); Arg, 5.0 (8); Cys, Trp, decomposed. Since rIFN- $\gamma$  contains 20 Lys residues, the above results indicate that about 12% of the Lys  $\epsilon$ -amino groups in rIFN- $\gamma$  had been modified by poly-  
5 ethylene glycol methyl ether (average molecular weight 750). The product had an antiviral activity of  $1.3 \times 10^6$  international units/mg. Administration of the product to rats resulted in obvious delay in clearance in blood. On the other hand, the precipitate was dissolved in 6 M guanidine  
10 hydrochloride and dialyzed against 25 mM ammonium acetate (pH 6.0) + 0.15 M sodium chloride + 10 mM glutathione at 4°C overnight, followed by Sephadex G-75 gel filtration in the same manner as above. The thus-purified fraction (25 ml) had a protein content of 126  $\mu$ g/ml and amino acid analysis of the  
15 acid hydrolysate (6 N hydrochloric acid, 110°C, 24 hours) gave the following values: Asp, 20.0 (20); Thr, 5.2 (5); Ser, 9.5 (11); Glu, 27.8 (18); Pro, 2.7 (2); Gly, 14.6 (5); Ala, 8.1 (8); Val, 8.5 (8); Met, 4.3 (4); Ile, 7.2 (7); Leu, 10.2 (10); Tyr, 5.8 (5); Phe, 10.1 (10); Lys, 14.7;  
20 His, 2.0 (2); Arg, 7.3 (8); Thr, 0.7 (1); Cys, decomposed. The higher values for Glu and Gly than the theoretical are presumably due to contamination by glutathione. Since rIFN- $\gamma$  contains 20 Lys  $\epsilon$ -amino groups, the above results indicate that about 26.5% of the Lys  $\epsilon$ -amino groups in  
25 rIFN- $\gamma$  had been modified by polyethylene glycol methyl ether.

(ii) Using 225 mg of polyethylene glycol methyl ether

aldehyde having an average molecular weight of 750 and 120 mg of sodium cyanoborohydride, rIFN- $\gamma$  was treated in the same manner as (i) in the presence of 2-mercaptopropanoic acid (2%) to give 30 ml of a polyethylene glycol methyl ether-modified rIFN- $\gamma$  solution having a protein content of 236  $\mu$ g/ml. Amino acid analysis of the acid hydrolysate (6 N hydrochloric acid, 110°C, 24 hours) gave the following values:

Asp, 20.0 (20); Thr, 5.2 (5); Ser, 9.6 (11); Glu, 33.6 (18); Pro, 1.8 (2); Gly, 19.9 (5); Ala, 8.2 (8); Val, 8.9 (8); Met, 4.6 (4); Ile, 7.4 (7); Leu, 10.2 (10); Tyr, 5.9 (5); Phe, 10.7 (10); Lys, 10.2; His, 2.3 (2); Arg, 7.9 (8); Trp, 0.6 (1); Cys, decomposed. The higher values for Glu and Gly are presumably due to contamination with glutathione. Since rIFN- $\gamma$  contains 20 Lys  $\epsilon$ -amino groups, the above results indicate that about 50% of the Lys  $\epsilon$ -amino groups in rIFN- $\gamma$  had been modified by polyethylene glycol methyl ether.

Example 5 Production of polyethylene glycol methyl ether-modified IFN- $\gamma$ d2

(i). A 5-ml portion (4.95 mg as protein) of the IFN- $\gamma$ d2 solution obtained in Reference Example 3 is applied to a Sephadex G-25 column (2.0 x 60.0 cm) and developed with 0.2 M phosphate buffer (pH 7.0). The eluate is fractionated by 5 ml. Fractions Nos. 11-13 are combined and diluted to 100 ml with the same buffer. To the dilution is added polyethylene glycol methyl ether aldehyde (average molecular weight 750) (200 mg), and then sodium cyanoborohydride (300 mg). The mixture is shaken at 37°C for 72 hours. The

resulting precipitate is removed by centrifugation. The supernatant is concentrated to 10 ml using a Diaflow membrane (Amicon). The concentrate is applied to a Sephadex G-75 column (3.0 x 43.0 cm) and developed with 5 25 mM ammonium acetate buffer (pH 6.0) + 0.15 M sodium chloride + 10 mM glutathione. The eluate is fractionated by 5 ml, and the fractions containing modified IFN- $\gamma$ d2 having the polyethylene glycol methyl ether moiety on the Lys  $\epsilon$ -amino group in the molecule are collected and combined. When this product is administered to rats, evident 10 delay in clearance in blood is noted.

On the other hand, the precipitate is dissolved in 6 M guanidine hydrochloride, dialyzed against 25 mM ammonium acetate buffer (pH 6.0) + 0.15 M sodium chloride + 10 mM 15 glutathione at 4° C overnight, and purified by Sephadex G-75 gel filtration in the same manner as above. Thus is obtained a fraction containing modified IFN- $\gamma$ d2 having the polyethylene glycol methyl ether moiety on the Lys  $\epsilon$ -amino group in the molecule.

Example 6 Production of polyethylene glycol methyl ether-  
20 modified IFN- $\gamma$ d3

(i) A 5-ml (5.5 mg as protein) portion of the IFN- $\gamma$ d3 solution obtained in Reference Example 4 is applied to a Sephadex G-25 column (2.0 x 60.0 cm), followed by development with 0.2 M phosphate buffer (pH 7.0). The eluate is 25 fractionated in 5-ml portions. Fractions Nos. 11-13 are combined, and thereto are added polyethylene glycol methyl-ether aldehyde (average molecular weight 750) (225 mg) and

then sodium cyanoborohydride (120 mg). The mixture is shaken at 37°C for 24 hours. The reaction mixture is applied to a Sephadex G-75 column (3.0 x 43.0 cm), followed by development with 25 mM ammonium acetate buffer (pH 6.0).

5 Thus is obtained a fraction containing modified IFN-γd3 with the polyethylene glycol methyl ether moiety on the Lys ε-amino group in the molecule. When this product is administered to rats, obvious delay in clearance in blood is observed.

10 Example 7 Production of polyethylene glycol methyl ether-modified IL-2

(i) A 5-ml (5.0 mg as protein) portion of the interleukin 2 (hereinafter abbreviated as rIL-2) obtained in Reference Example 5 was dialyzed against 0.2 M phosphate buffer (pH 15 7.15) for 12 hours. To the dialyzate was added polyethylene glycol methyl ether aldehyde (average molecular weight 750) (97 mg), and then sodium cyanoborohydride (100 mg). The mixture was stirred at 37°C for 24 hours. The resultant precipitate was removed by centrifugation. The 20 supernatant was dialyzed against 5 mM ammonium acetate buffer (pH 5.0) for 5 hours. The dialyzate was applied to a Sephadex G-75 column (3.0 x 43.0 cm) and developed with the same solvent system. The eluate was fractionated in 5-ml portions. The desired product-containing fractions 25 Nos. 21-29 were combined. The combined fraction had a protein content of 25 µg/ml as determined by the Bradford method using bovine serum albumin as a standard. The acid

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hydrolysate (6 N hydrochloric acid, 110°C, 24 hours) gave the following amino acid analysis values: Asp, 12.0 (12); Thr, 12.5 (13); Ser, 7.1 (8); Gly, 18.6 (18); Pro, 5.5 (5); Gly, 2.2 (2); Ala, 5.0 (5); Val, 3.7 (4); Met, 3.9 (4); Ile, 8.1 (8); Leu, 22.2 (22); Tyr, 3.0 (3); Phe, 6.0 (6); Lys, 7.3; His, 3.0 (3); Arg, 3.9 (4); Cys, Trp, de-composed. Since rIL-2 contains 11 Lys residues, the above results indicate that about 33.6% of the Lys ε-amino groups had been modified by polyethylene glycol methyl ether. The IL-2 activity of the product as determined by the method of Hinuma et al. [Biochemical and Biophysical Research Communications, 109, 363-369 (1982)] which measures the growth of an IL-2-dependent mouse natural killer cell line (NKC3) with the [<sup>3</sup>H]-thymidine uptake into DNA as an index was 15 22,998 units/mg. When rIL-2 is supposed to have an activity of 40,000 units/mg, the product is estimated to retain 57.7% of the activity. After administration of this product, obvious delay in clearance in blood was noted.

Reference Example 1 Synthesis of polyethylene glycol methyl-ether aldehyde

(i) Polyethylene glycol methyl ether (5 g; average molecular weight 5,000) was dissolved in methylene chloride (100 ml) and then pyridinium chlorochromate (330 mg) was added. The mixture was stirred at room temperature for 12 hours. The reaction mixture was diluted two-fold with methylene chloride and poured into a Florisil column (6 x 10 cm), and the column was washed with methylene chloride and then with chloroform, followed by elution with methanol-chloroform (1:9). Fractions positive to 2,4-dinitrophenyl-hydrazone test were combined, the solvent was distilled off under reduced pressure, and there was obtained a crystalline wax. Yield 1.5 g (30%). Thin layer chromatography:  $R_f = 0.08$  (chloroform-methanol-acetic acid = 9:1:0.5, silica gel).

$^{13}\text{C-NMR}$  spectrometry revealed an absorption due to the aldehyde group in hydrated form ( $-\text{CH}(\text{OH})_2$ ) at 96.2 ppm.

(ii) Polyethylene glycol methyl ether (10 g; average molecular weight 5,000) was dissolved in tertiary-butanol (100 ml). Thereto was added potassium tertiary-butoxide (4.17 g), followed by addition of bromoacetal (2.56 ml). The mixture was stirred at 40°C for 2 hours. The tertiary-butanol was then distilled off under reduced pressure,

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water was added to the residue, and the aqueous mixture was extracted with chloroform (200 ml x 2). The extract was washed with water and dried over anhydrous sodium sulfate. The chloroform was then distilled off under reduced pressure, 5 petroleum benzine was added to the residue, and the resultant crystalline residue was collected by filtration and washed with ether. Thus was obtained 9.5 g (95%) of the corresponding polyethylene glycol methyl ether diethyl acetal. A 5-g portion of the acetal was dissolved in 50 ml 10 of 0.05 M trifluoroacetic acid, treated in a boiling water bath for 30 minutes and then lyophilized, giving a polyethylene glycol methyl ether aldehyde longer in chain length by  $-O-CH_2CH_2-$  than the product obtained in (i).

(iii) Polyethylene glycol methyl ether (5.7 g; average 15 molecular weight 1,900) was dissolved in methylene chloride (100 ml) and then pyridinium chlorochromate (970 mg) was added. The mixture was stirred at room temperature for 12 hours, then diluted with an equal volume of methylene chloride, and poured into a Florisil column (6.0 x 10.0 cm). 20 The column was washed with methylene chloride and then with chloroform, followed by elution with 10% methanol/chloroform. Fractions positive to 2,4-dinitrophenylhydrazine test were combined. Removal of the solvent by distillation gave a crystalline wax. Yield 1.8 g (30%). Thin layer chromatography:  $R_f = 0.10$  (chloroform-methanol-acetic acid = 9:1:0.5, 25 silica gel).  $^{13}C$ -NMR spectrometry indicated the presence of an absorption due to the aldehyde group in hydrated form

(-CH(OH)<sub>2</sub>) at 96.2 ppm.

(iv) Polyethylene glycol methyl ether (19.5 g; average molecular weight 1,900) was dissolved in tertiary-butanol (100 ml). Potassium tertiary-butoxide (10.4 g) was added and then bromoacetal (6.4 ml) was added. The mixture was stirred at 40°C for 2 hours. The tertiary-butanol was then distilled off under reduced pressure. Water was added to the residue, followed by extraction with chloroform (200 ml x 2). The extract was washed with water and dried over anhydrous sodium sulfate. The chloroform was distilled off under reduced pressure. Petroleum benzine was added to the residue, and the resultant crystalline residue was collected by filtration and washed with ether to give 8.5 g (89.5%) of acetal. A 3-g portion of the acetal was dissolved in 0.05 M trifluoroacetic acid, and the solution was treated in a boiling water bath for 30 minutes and then lyophilized to give a polyethylene glycol methyl ether aldehyde longer in chain length by -O-CH<sub>2</sub>CH<sub>2</sub>- than the product obtained in (iii).

(v) Polyethylene glycol methyl ether species having average molecular weights of 750, 550 and 350 were derived to the corresponding aldehyde species by following the above procedures.

Reference Example 2 Synthesis of alkanoyl polyethylene-glycol aldehyde

(i) In 50 ml of pyridine, there was dissolved 15 g of polyethylene glycol 1540 (Wako Pure Chemical Industries)

5 (average molecular weight 1500). To the solution was added 1.85 ml of acetic anhydride. The mixture was stirred at 40°C for 2 hours and then at room temperature for 16 hours. Thereafter, the solvent was distilled off under reduced pressure. The residue was dissolved in chloroform, and the 10 solution was washed with water, the chloroform layer was dried over anhydrous sodium sulfate, and the chloroform was distilled off under reduced pressure. The residue was dissolved in a small amount of chloroform, a petroleum benzene-ether (2:1) mixture was added to the solution, and the mixture was allowed to stand 15 to give 14 g (90%) of a crystalline wax. A 1.4-g portion of the wax was dissolved in 50 ml of methylene chloride, followed by addition of 300 mg of pyridinium chlorochromate. The resulting mixture was stirred at room temperature for 18 hours. The reaction mixture was applied to a silica 20 gel C-200 (Wako Pure Chemical Industries) column (3 x 50 cm), and the column was washed with 5% methanol-chloroform (200 ml) and eluted with 10% methanol-chloroform. Fractions positive to the 2,4-dinitrophenylhydrazine test were combined, and the solvent was distilled off under reduced pressure. A crystalline wax was obtained. Yield 580 mg (41%).

25 (ii) In 50 ml of methylene chloride, there was dissolved 20 g of polyethylene glycol 1000 (Wako Pure Chemical Ind.)

(average molecular weight 1000), followed by addition of 5.15 g of n-caproyl anhydride. The mixture was stirred at 70°C for 2 hours. Then, the solvent was distilled off, and the residue was purified using a silica gel C-200 column (3 x 5 50 cm) and elution with ethyl acetate-methanol (4:1) to give 14.9 g (60%) of an oil, which solidified upon standing in a refrigerator. The subsequent oxidation with pyridinium chlorochromate as conducted in the same manner as (i) gave the corresponding aldehyde.

10 Reference Example 3 Production of IFN-γd2

(i) Transformant preparation

The IFN-γ expression plasmid pHITtrpl101 [cf. EPC (laid open) No. 110044, Example 2 (iii)] was digested with the restriction enzymes AvaII and PstI, and an AvaII-PstI 1 kb DNA fragment containing the IFN-γ gene portion was isolated. 15 The protein synthesis start codon-containing oligonucleotide adapter

CGATAATGTGCCAG

TATTACACGGTCCTG

20 chemically synthesized by the phosphotriester method was joined to the above DNA fragment at the AvaII cohesive end thereof using T4 DNA ligase.

The above adapter-joined gene was inserted into the DNA fragment obtained by cleavage of the plasmid pptrp771 25 [cf. above-cited publication, Example 2 (ii)] with the restriction enzymes ClaI and PstI, downstream from the trp promoter in said fragment. Thus was constructed the ex-

pression plasmid pHITtrpl101-d2 coding for the Cys-Tyr-  
deficient IFN- $\gamma$  polypeptide (Fig. 3).

Escherichia coli 294 was transformed with this plasmid  
pHITtrpl101-d2 by the method of Cohen et al. [Proc. Natl.  
5 Acad. Sci. U.S.A., 69, 2110 (1972)] to give the transformant  
Escherichia coli (=E. coli) 294/pHITtrpl101-d2 carrying  
said plasmid.

(ii) Transformant cultivation

The strain E. coli 294/pHITtrp 1101-d2 carrying the plasmid  
10 constructed in (i) above was cultivated in M9 medium con-  
taining 8  $\mu$ g/ml of tetracycline, 0.4% of casamino acids  
and 1% of glucose at 37°C. When the growth reached KU 220,  
3- $\beta$ -indolylacrylic acid (IAA) was added to a concentration  
of 25  $\mu$ g/ml. Thereafter, the cultivation was continued  
15 for further 4 hours. After cultivation, cells were har-  
vested by centrifugation and suspended in 1/10 volume of  
0.05 M Tris-HCl (pH 7.6) containing 10% sucrose. To the  
suspension, there were added phenylmethylsulfonyl fluoride,  
NaCl, ethylenediaminetetraacetate (EDTA), spermidine and  
20 lysozyme to concentrations of 1 mM, 10 mM, 40 mM and 200  
 $\mu$ g/ml, respectively. After standing at 0°C for 1 hour,  
the suspension was treated at 37°C for 3 minutes to give  
a lysate.

The lysate was subjected to centrifugation at 4°C  
25 and 20,000 rpm (Servall centrifuge, SS-34 rotor) for 30  
minutes to give an IFN- $\gamma$ d2 polypeptide-containing super-  
natant. This supernatant had an antiviral activity of

$2.87 \times 10^8$  U/liter culture fluid.

(iii) Purification of IFN- $\gamma$ d2

In 18 ml of 0.1 M Tris-hydrochloride buffer (pH 7.0) containing 7 M guanidine hydrochloride and 2 mM phenyl-methylsulfonyl fluoride, there were suspended 5.9 g of cells obtained in the same manner as (ii) above and stored in the frozen state. The suspension was stirred at 4°C for 1 hour and then subjected to centrifugation at 10,000  $\times$  g for 30 minutes to give 20 ml of a supernatant. This supernatant was diluted with 260 ml of a buffer (pH 7.4) comprising 137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM disodium phosphate and 1.5 mM monopotassium phosphate (hereinafter such buffer being referred to by the abbreviation PBS) and the dilution was applied to an antibody column (Mo $\gamma$ 2-11.1, column volume 12 ml) at a flow rate of 1 ml/minute. The column was then washed with 60 ml of 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M guanidine hydrochloride and eluted with 36 ml of 20 mM sodium phosphate buffer (pH 7.0) containing 2 M guanidine hydrochloride to give 20 ml of an antivirally active fraction.

This 20-ml fraction was applied to a Sephadryl S-200 (Pharmacia) column (2.6 x 94 cm, column volume 500 ml) equilibrated in advance with 25 mM ammonium acetate buffer (pH 6.0) containing 1 mM ethylenediaminetetraacetate, 0.15 M sodium chloride, 10 mM cysteine and 2 M guanidine hydrochloride, followed by elution with the same buffer. Thus was obtained 37 ml of an antivirally active fraction.

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The Cys-Tyr-deficient IFN- $\gamma$  polypeptide (IFN- $\gamma$ d2)  
obtained weighed 5.9 mg and had a specific activity of  
 $1.0 \times 10^7$  U/mg.

Reference Example 4 Production of IFN-γ d3

(i) Transformant production

The IFN-γ expression plasmid pRC23/IFI-900 [cf. Example 7 of the specification for a patent application under EPC as laid open under No. 0089676] was digested with the restriction enzymes NdeI and NcoI, and a 710 bp NdeI-NcoI DNA fragment (A) containing the IFN-γ gene region was isolated. Separately, the plasmid pRC23 was digested with the restriction enzymes BclII and EcoRI, and a 265 bp DNA fragment (B) containing the λP<sub>L</sub> promoter was isolated.

The fragments (A) and (B) and the chemically synthesized, protein synthesis start codon-containing oligonucleotide

AATTCAATGCAGGRTCCA

GTAACGTCCTTGGGTAT

were joined together using T4 DNA ligase, with the NdeI and EcoRI cohesive ends as the sites of joining. The DNA fragment thus obtained was joined to the plasmid pRC23/IFI-900 after treatment with NcoI and BclII, to thereby construct an expression plasmid, pLC2, coding for the Cys-<sup>2</sup>-Cys-<sup>3</sup>-Ty<sup>z</sup>-Cys-deficient IFN-γ polypeptide (Fig. 2). This plasmid pLC2 was used for transforming Escherichia coli RRI(pRK248 cIts) by the method of Cohen et al. [supra] to give a transformant, Escherichia coli (=E. coli) PRI(pLC2,pRK248 cIts).

(ii) Transformant cultivation

The strain E. coli RRI(pLC2,pRK248 cIts) carrying the plasmid constructed in (i) above was shake-cultured

at 35°C in 50 ml of a liquid medium containing 1% Bacto-tryptone, 0.5% yeast extract, 0.5% sodium chloride and 7 µg/ml tetracycline. The culture broth was transferred to 2.5 liters of M9 medium containing 0.5% casamino acid, 5 0.5% glucose and 7 µg/ml tetracycline, and grown at 35°C for 4 hours and then at 42°C for 3 hours. Cells were harvested by centrifugation and stored at -80°C.

(iii) Purification

In 22 ml of 0.1 M Tris-hydrochloride buffer (pH 7.0) containing 7 M guanidine hydrochloride and 2 mM phenyl-methylsulfonyl fluoride, there were suspended 7.1 g of frozen cells obtained in the same manner as mentioned above in (ii). The suspension was stirred at 4°C for 1 hour and then centrifuged at 10,000 x g for 30 minutes 15 to give 24 ml of a supernatant. This supernatant was diluted by adding 300 ml of PBS and the dilution was applied to an antibody column (Moy2-11.1, column capacity 15 ml) at a flow rate of 1 ml/minute. Thereafter, the column was washed with 60 ml of 20 mM 20 sodium phosphate buffer (pH 7.0) containing 0.5 M guanidine hydrochloride and then eluted with 45 ml of 20 mM sodium phosphate buffer (pH 7.0) containing 2 M guanidine hydrochloride, to give 25 ml of an antivirally active fraction. This fraction (25 ml) was applied to a Sephadex S-200 25 (Pharmacia) column (2.6 x 94 cm; column capacity 500 ml)

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equilibrated in advance with 25 mM ammonium acetate buffer  
(pE 6.0) containing 1 mM ethylenediaminetetraacetic acid,  
0.15 M sodium chloride, 10 mM cysteine and 2 M guanidine  
hydrochloride, and eluted with the same buffer to give  
5 40 ml of an antivirally active fraction.

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The thus-obtained Cys-Tyr-Cys-deficient IFN- $\gamma$  poly-  
peptide IFN- $\gamma$  d3 weighed 7.0 mg and had a  
specific activity of  $2.72 \times 10^7$  IU/mg.

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Reference Example 5 Production of non-glycosylated human IL-2

(i) Transformant cultivation.

E. coli DH1/pTF4 [ EPC Pat. Appln. No. 84 308153, D ]

was inoculated

5 into 50 ml of a liquid medium (pH 7.0) containing 1% Bacto  
tryptone (Difco Laboratories, USA), 0.5% Bacto yeast extract  
(Difco Laboratories, USA), 0.5% sodium chloride and 7 ug/ml  
tetracycline as placed in a 250-ml Erlenmeyer flask. After  
incubation at 37°C overnight on a swing rotor, the culture  
10 medium was transferred to a 5-liter jar fermenter containing

2.5 liters of M9 medium containing 0.5% casamino acid, 0.5% glucose and 7  $\mu$ g/ml tetracycline. Incubation was then conducted with aeration and stirring at 37°C for 4 hours and, after addition of 3- $\beta$ -indolylacrylic acid (25  $\mu$ g/ml), for 5 further 4 hours. Cells were harvested from the thus-obtained 2.5-liter culture broth by centrifugation, frozen at -80°C and stored.

(ii) Extraction

The freeze-stored cells (12.1 g) obtained above 10 were suspended uniformly in 100 ml of an extractant (pH 7.0) containing 7 M guanidine hydrochloride and 0.1 M Tris-HCl, the suspension was stirred at 4°C for 1 hour and the lysate was centrifuged at 28,000  $\times$  g for 20 minutes. There was obtained 93 ml of a supernatant.

15 (iii) Purification of IL-2 protein

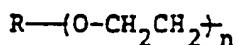
The supernatant obtained above was dialyzed against 0.01 M Tris-HCl buffer (pH 8.5) and then centrifuged at 19,000  $\times$  g for 10 minutes, giving 94 ml of a dialyzate supernatant. This dialyzate supernatant was applied to 20 a DE 52 (DEAE-cellulose, Whatman, Great Britain) column (50 ml in volume) equilibrated with 0.01 M Tris-HCl buffer (pH 8.5) for protein adsorption. IL-2 was eluted making a linear NaCl concentration gradient (0-0.15 M NaCl, 1 liter). The active fractions (53 ml) were concentrated to 4.8 ml 25 using a YM-5 membrane (Amicon, USA) and subjected to gel filtration using a Sephadryl S-200 (Pharmacia, Sweden) column (500 ml in volume) equilibrated with 0.1 M Tris-HCl.

(pH 8.0)-1 M NaCl buffer. The active fractions (28 ml) obtained were concentrated to 2.5 ml using a YM-5 membrane. The concentrate was applied to a Ultrapore RPSC (Altex, USA) column for adsorption, and high performance liquid chromatography was performed using a trifluoroacetic acid-acetonitrile system as the eluent.

Under the conditions: column, Ultrapore RPSC (4.6 x 75 mm); column temperature, 30°C; eluent A, 0.1% trifluoroacetic acid-99.9% water; eluent B, 0.1% trifluoroacetic acid-  
10 99.9% acetonitrile; elution program, minute 0 (68% A + 32% B) - minute 25 (55% A + 45% B) - minute 35 (45% A + 55% B) - minute 45 (30% A + 70% B) - minute 48 (100% B); elution rate, 0.8 ml/min.; detection wave length, 230 nm. An active fraction was collected at a retention time of about 39 minutes.  
15 Thus was obtained 10 ml of a solution containing 0.53 mg of non-glycosylated human IL-2 protein [specific activity, 40,000 U/mg; activity recovery from starting material, 30.6%; purity of protein, 99% (determined by densitometry)].

What is claimed is:

1. A chemically modified lymphokine having polyethylene glycol of the formula:



wherein R is a protective group for the terminal oxygen atom and n is an optional positive integer, bonded directly to at least one primary amino group of the lymphokine moiety.

2. The modified lymphokine according to claim 1, wherein the lymphokine moiety has molecular weight from 5,000 to 50,000.

3. The modified lymphokine according to claim 2, wherein the lymphokine moiety has molecular weight from 10,000 to 30,000.

4. The modified lymphokine according to claim 1, wherein the lymphokine moiety is interferons, interleukin-2, macrophage differentiating factor, macrophage activating factor, or substances similar in structure and in physiological activity to these.

5. The modified lymphokine according to claim 1, wherein the lymphokine moiety is interferon- $\alpha$ , interferon- $\beta$ , interferon- $\gamma$ , interferon- $\gamma d2$ , interferon- $\gamma d3$  or interleukin-2.

6. The modified lymphokine according to claim 1, wherein the lymphokine moiety is interferon- $\alpha$ .

7. The modified lymphokine according to claim 1, wherein the lymphokine moiety is interferon- $\gamma$ .

8. The modified lymphokine according to claim 1, wherein the lymphokine moiety is interleukin-2.

9. The modified lymphokine according to claim 1, wherein the polyethylene glycol has molecular weight corresponding to 1 to 10% of the molecular weight of the lymphokine moiety.

10. The modified lymphokine according to claim 1, wherein the polyethylene glycol has molecular weight from 350 to 6,000.

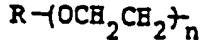
11. The modified lymphokine according to claim 1, wherein R is alkyl or alkanoyl.

12. The modified lymphokine according to claim 1, wherein n is a positive integer from 7 to 120.

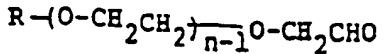
13. The modified lymphokine according to claim 1, wherein the primary amino group is N-terminal  $\alpha$ -amino group or  $\epsilon$ -amino group of lysine residue in the lymphokine moiety.

14. The modified lymphokine according to claim 1, which has polyethylene glycol bonded to 15 to 80% of  $\epsilon$ -amino groups of lysine residue in the lymphokine moiety.

15. A method of producing a chemically modified lymphokine having polyethylene glycol of the formula:



wherein R is a protective group for the terminal oxygen atom and n is an optional positive integer, bonded directly to at least one primary amino group of the lymphokine moiety, which comprises reacting a lymphokine with an aldehyde of the formula:



wherein R and n are as defined above, in the presence of a reducing agent.

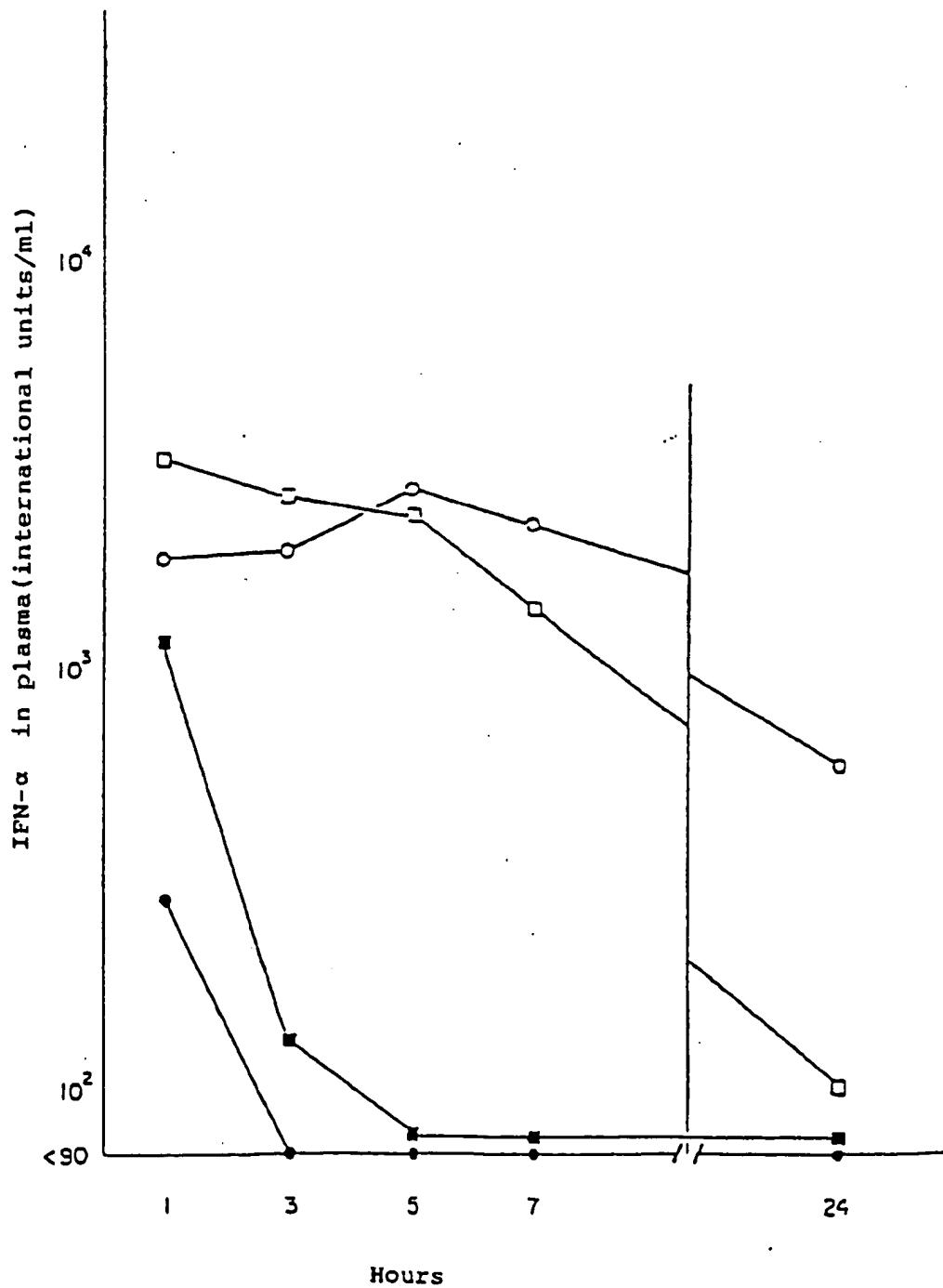
16. The method according to claim 15, wherein the reaction is conducted in the neighborhood of neutrality.

17. The method according to claim 15, wherein the reducing agent is sodium cyanoborohydride.

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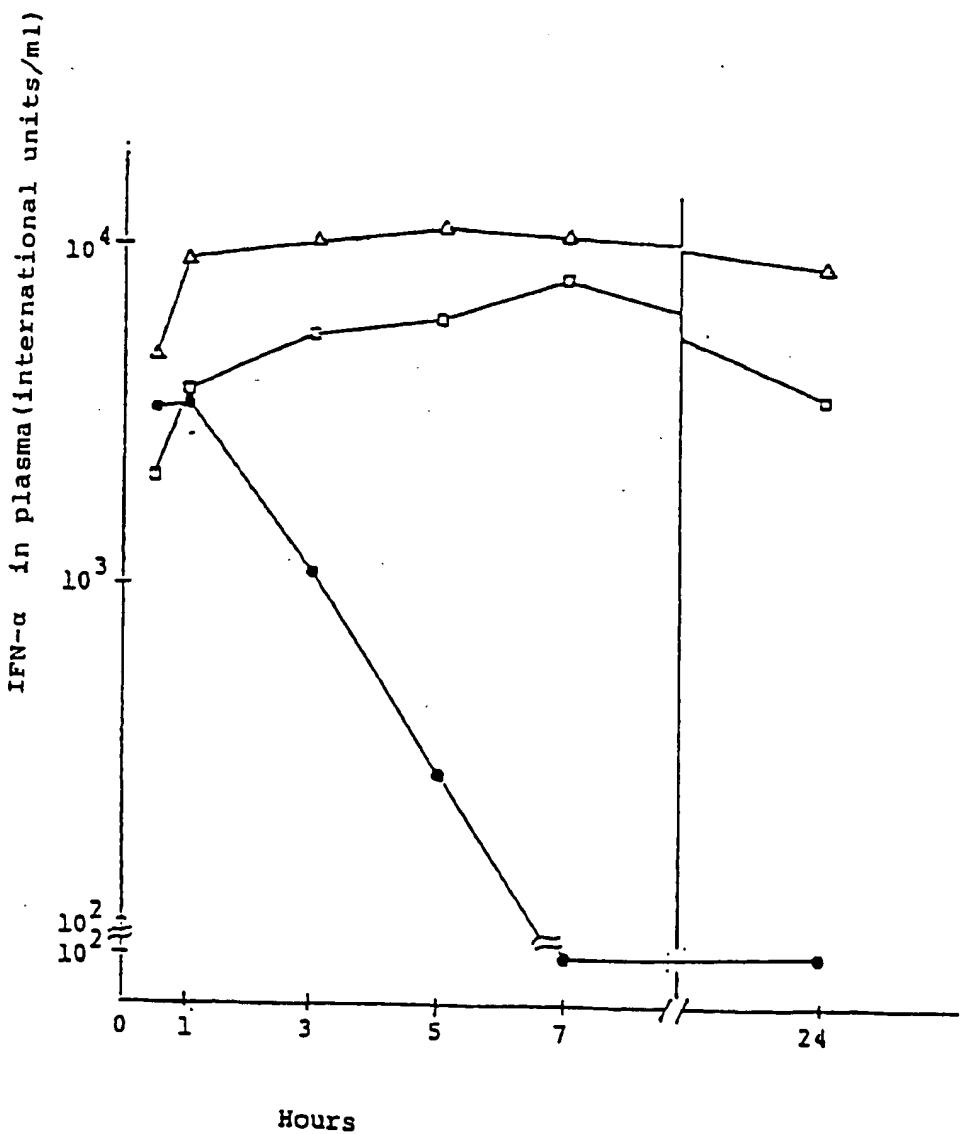
Fig. 1



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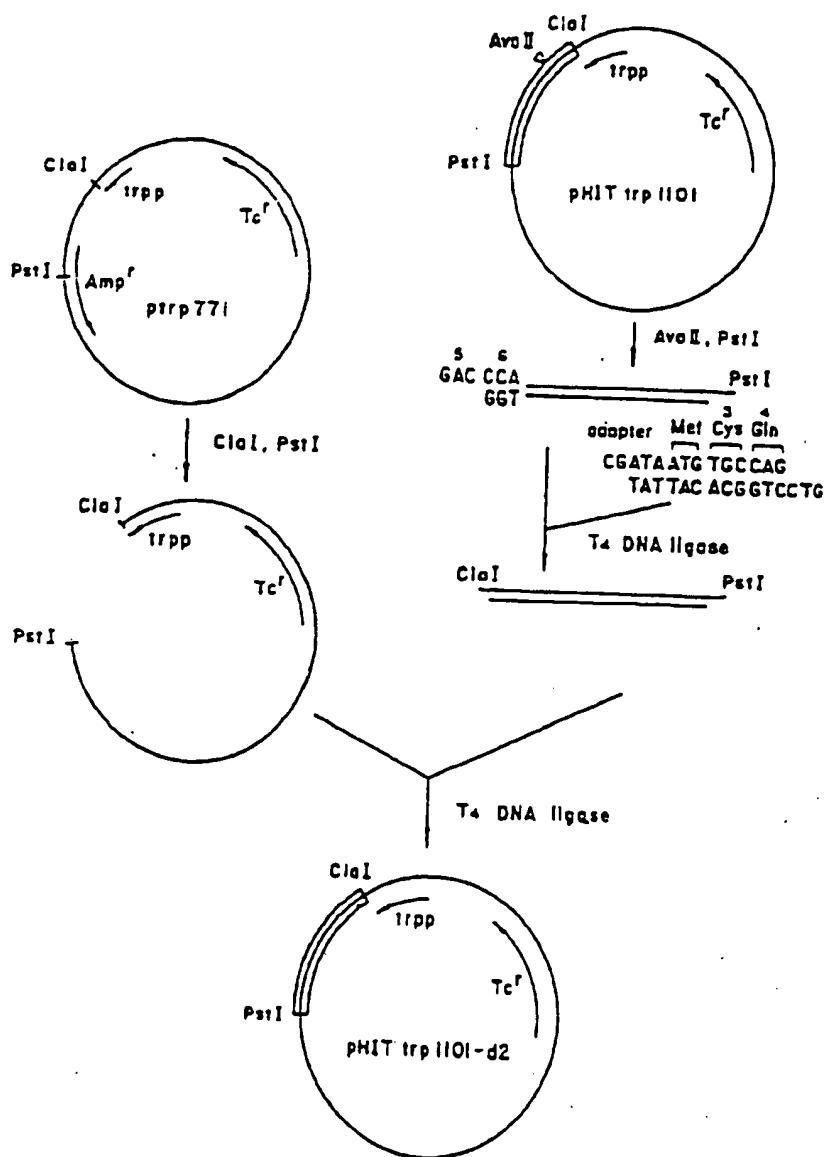
Fig. 2



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Fig. 3



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Fig. 4

